107. FAT-DEFICIENCY DISEASE OF RATS. THE EFFECT OF DOSES OF METHYL ARACHIDONATE AND LINOLEATE ON FAT METABOLISM, WITH A NOTE ON THE ESTIMATION OF ARACHIDONIC ACID

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A HIGHLY unsaturated acid containing 20 carbon atoms and four double bonds was isolated by Hartley [1909] from the fatty acids of the pig's liver as its octabromide and named by him arachidonic acid. Subsequently the same acid was shown by Levene & Rolf [1922] to occur as a constituent of lecithin and kephalin, and since that time it has been shown to be widely distributed in animal tissues. In the ox, it was identified among the fatty acids of the liver, both in the neutral fat and in the phospholipin fraction; it occurs also in the acids from the fat depots and in the phospholipins of the heart, spleen and adrenals [Klenk & v. Schoenebeck, 1932], whilst Klenk & Dittmar [1936] and Wesson [1924] identified it among the brain fatty acids. Wesson [1925] estimated its proportion in the various tissues and endeavoured to determine the part played by this acid in metabolic processes.

Burr & Burr [1930] showed that the presence of highly unsaturated acids was essential for normal growth in rats and that these acids were not synthesized in the animal organism but had to be supplied in the diet. They found that either linoleic or linolenic acid could provide the missing factor, and that when either of these was included in the diet, normal growth was resumed. Wesson [1925] in his experiments had used corn starch as a constituent of the fat-deficient diet: this however on hydrolysis with acids yields appreciable quantities of linoleic acid [Taylor & Nelson, 1920] so that the essential fatty acid was actually being included in the diet of these fat-starved rats. It was also then not known how very slowly the body is depleted of the essential acids and that only very long-continued experiments will produce the symptoms of fat deficiency.

Burr *et al.* [1932] replaced 10% of a mixture of linoleate and linolenate by arachidonate and found that the relative increase of weight of rats receiving this mixture diminished when compared with rats receiving the full linoleate and linolenate supplements. It is however probable that the effect of a 10% substitution would give results within the biological variation of the experiment.

Turpeinen [1938] found methyl arachidonate to be three times as potent in producing weight increase as methyl linoleate; he did not specifically describe its effect on skin symptoms in the different rats but, speaking generally, said that the skin symptoms met with in the laboratory were mild. He measured however the number of ovulation cycles and found these to be markedly less than normal in the fat-starved rats but to be increased when arachidonate or

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linoleate was fed. He put forward the tentative hypothesis that the primary need of the body was for arachidonic acid.

Hume *et al.* [1938] showed that methyl linoleate was much more effective as a curative agent than methyl linolenate and Nunn & Smedley-MacLean [1938] established that arachidonic acid was not present in the liver fat of rats fed on a fat-free diet; when however the fat-starved rats received daily doses of methyl linoleate for a period of 5 weeks, arachidonic acid again appeared in the liver fat.

Burr *et al.* [1939] stated that they had found linoleic acid to be more useful in curing and preventing fat deficiency than either linolenic, arachidonic or the other higher unsaturated acids in fish oils.

Having in co-operation with E. M. Hume and H. H. Smith determined the effects of supplements of arachidonate on the growth and on the skin-condition of rats after a long period on a fat-free diet, we proceeded to investigate the fat of these rats. In our first experiment we examined the livers, kidneys and samples of muscle of four groups of these rats, determining the total content of lipoid matter, the percentage of phospholipin and the amounts of arachidonic acid measured by the weight of benzene-insoluble bromide formed on bromination in the phospholipin and neutral fat respectively.

Twenty-nine rats whose growth has been already described [Hume *et al.* 1940] were divided into 4 groups:

Group I. Eight rats which for 216 days after weaning had received only the fat-free diet.

Group II. Seven rats, of which after 163 days of the fat-free diet three received a supplement of 42 mg. and four, one of 14 mg. linoleate daily.

Group III. Six rats which, after receiving the fat-free diet for 163 days, then were given in addition, a supplement of 14 mg. methyl arachidonate for 39 days before being killed.

Group IV. Eight rats, six of which after the 163rd day of the fat-free diet received a daily supplement of 14 mg. arachidonate and two of which received daily 42 mg. arachidonate for 47 days before being killed.[1]

Method of preparation of phospholipin. Immediately after death, the livers, kidneys and samples of muscle from the lower limbs and body-wall were removed and the similar organs of each set of rats worked up together. The organs were sliced and dehydrated by immersion in acetone for 24 hr.; the dried residue was then twice extracted, each time for 1 hr. with boiling alcohol in an atmosphere of N_2 and finally with ether in a Soxhlet apparatus until no more fat could be extracted. The solvents were removed under diminished pressure and the residues extracted with ether. The ethereal solution was concentrated and after drying with anhydrous Na_2SO_4 was precipitated with acetone to remove the phospholipin fraction.

The degree of purification of the phospholipin varied with the amount available. When there was sufficient, it was precipitated four times by acetone from ethereal solution, the precipitate twice emulsified with a few drops of dilute NaCl solution and precipitated from this by acetone according to the method described by H. MacLean [1914]. The centrifuge was used to separate the precipitates and the ethereal solution was finally centrifuged to remove any deposit. The division into neutral fat and phospholipin is not an accurately quantitative separation but the results are comparative and should show any marked differences. Where the percentage of phospholipin was very small the neutral fat after the first separation of phospholipin was again dissolved in ether, and excess of acetone added to ensure complete separation. If the quantity of ether used as solvent had been small and the acetone in considerable excess, the amounts of phospholipin separated by reprecipitation were negligible. The degree of purity of the phospholipin was checked by phosphorus determination.

[1] The rats in groups I, II and IV, 4 hr. before being killed each received an injection of radioactive phosphate supplied by Prof. Hevesy.

Estimation of arachidonic acid. After saponification of neutral fat or phospholipin by boiling for 1 hr. with 10% alcoholic KOH, a current of nitrogen being passed during this operation, the free acids were extracted with ether and the ethereal solution dried overnight with anhydrous Na_2SO_4; the residue from the ethereal solution was dissolved in benzene and bromine carefully added to the ice-cooled solution.

After standing overnight at 0, the insoluble bromide which had separated was filtered off, washed with a few drops of benzene and then with ether until the washings gave only a trace of solid when a drop was evaporated on a watch-glass. The insoluble residue was then heated with 5 ml. benzene in a centrifuge tube in a water-bath at about 80°, the mixture centrifuged, the benzene solution decanted and the process repeated two or three times until the supernatant liquid contained only a trace of solid. Finally the residue after decanting the solution was washed with a little ether, centrifuged, the ether poured off and the centrifuge tube with the insoluble bromide dried to constant weight in a vacuum desiccator. Where the weight of bromide precipitate was more than about 12 mg. the percentage of Br was determined.

Although only about one-sixth of the theoretical amount of octabromide was precipitated when arachidonic acid, prepared by the debromination of the ether-insoluble bromide, was brominated in benzene solution of approximately 2% concentration, this proportion was found to vary but slightly and twice the weight of crude bromide 2 gave a very fair approximation to the amount of arachidonic acid present. Applying this to the mixed acids from the crude suprarenal fat and working at varying dilutions of from about 2% to 0·1% of arachidonic acid, we obtained reasonably constant results.

On washing with benzene as described in the method of estimation given above, about one-third of the precipitate was removed, and we felt it was justifiable to take the amount of arachidonic acid present as being approximately represented by 3 times the weight of benzene-insoluble bromide.

On further investigation we have found that the natural and debrominated acids show marked differences in the solubilities of their bromides and these results are discussed in the final section of this paper. We have therefore given the actual weights of benzene-insoluble bromides obtained under as far as possible comparable conditions. These weights probably represent about one-third the weights of arachidonic acid present. The method, whilst fairly satisfactory in the case of the natural acid, will give a low figure where the mixed fatty acids contain a small proportion of the debrominated acid which has been fed to the rats.

Results of exp. 1

The results of the analyses are shown in Table 1, Groups I–IV. The proportions of fat contained in liver, muscle and kidney tissues agreed closely in all four groups of rats, the percentages calculated on the weights of wet tissue lay in the kidney and liver between 3·1 and 3·5%: in muscle between 3·5 and 4·0%.

No significant variation in the proportions of phospholipin and neutral fat occurred: the values in the rats receiving only the fat-free diet were similar to those in the animals which had received the supplements of unsaturated acids. In most cases the lipoid matter from the livers contained approximately 50% phospholipin and 50% neutral fat, the greatest variation being between the two groups which had both received supplements of arachidonate but for varying periods. In the muscle the proportion of phospholipin in the lipoid matter varied from 6 to 13% but the differences could not be correlated with the presence or absence of the unsaturated acids.

The proportion of phospholipin in the kidney fat of the rats which had received the arachidonic acid supplement was much lower, but the amounts analysed were very small and this result would require further confirmation before it could be regarded as significant.

Amounts of arachidonic acid present in the organs of fat-starved rats. Bromination of the phospholipin acids derived from the liver of the negative controls

Table 1. *Showing the amounts of fat and phospholipin in organs of rats on various diets*

Group

I. 215 days on fat-free diet alone

II. 163 days on fat-free diet alone
+60 days with 1 drop linoleate daily

III. 163 days on fat-free diet
+39 days with 1 drop arachidonate daily

IV. 163 days on fat-free diet
+47 days with 1 drop arachidonate daily

V. 163 days on fat-free diet alone
+35 days with 1 drop arachidonate daily
+14 days on fat-free diet alone

Group

VI. 163 days on fat-free diet alone
+35 days with ½ drop arachidonate daily
+31 days on fat-free diet alone

VII. 163 days on fat-free diet alone
+35 days on ½ drop arachidonate daily
+40 days on fat-free diet alone

VIII. 163 days on fat-free diet alone
+35 days with 1 drop linoleate daily
+12 days on fat-free diet alone

Group	No. of rats	Wt. wet tissue g.	Wt. dry tissue fat-free g.	Wt. neutral fat g.	Wt. phospho-lipin g.	% P in phospho-lipin	% P lipin in total lipin	% total lipin in wet tissue
				Liver				
I (−ve)	8	61·8	—	1·00	1·00	3·55	50·0	3·24
II (1 L)	7	51·0	—	0·86	0·93	3·61	51·9	3·50
III (1 A)	6	46·0	—	0·69	0·87	3·12	55·8	3·40
IV (1 A)	8	61·0	—	1·00	0·92	3·39	44·6	3·40
V (1 A) (−ve)	4	35·0	—	0·70	0·41	3·23	36·9	3·17
VI (½ A) (−ve)	4	35·5	8·43	0·60	0·34	3·12	30·0	3·24
VII (¼ A) (−ve)	4	32·9	8·15	0·79	0·53	3·22	40·2	4·00
VIII (1 L) (−ve)	4	35·2	—	0·67	0·76	2·90	53·1	4·06
Wistar rats on normal diet:								
IX	4	25·8	5·17	1·29	—	—	—	5·00
X	4	24·2	5·06	0·87	—	—	—	3·60
				Muscle				
I (−ve)	8	98·9	—	3·24	0·41	3·02	11·2	3·7
II (1 L)	7	111·2	—	3·92	0·27	3·07	6·2	3·9
III (1 A)	6	100·7	—	3·04	0·46	2·90	13·1	3·5
IV (1 A)	8	114·5	—	4·28	0·34	2·79	6·6	4·0
V (1 A) (−ve)	4	52·5	—	2·31	0·25	2·70	9·8	4·9
VI (½ A) (−ve)	4	52·7	12·1	3·42	0·46	2·83	11·8	7·4
VII (¼ A) (−ve)	4	58·6	11·8	2·50	0·29	3·07	9·0	4·3
VIII (1 L) (−ve)	4	57·0	—	2·29	0·34	2·90	12·9	4·6
Wistar rats on normal diet:								
IX	4	40·6	9·7	—	—	—	—	3·0
X	4	30·1	6·8	—	—	—	—	4·5
				Kidney				
I (−ve)	8	17·5	—	0·34	0·20	2·77	37·0	3·08
II (1 L)	7	14·4	—	0·32	0·16	3·13	33·3	3·40
III (1 A)	6	—	—	—	—	—	—	—
IV (1 A)	8	17·2	—	0·44	0·10	3·28	18·5	3·14
V (1 A) (−ve)	4	7·8	—	0·35	0·04	1·74	10·2	5·62
VI (½ A) (−ve)	4	8·7	—	0·09	0·34	2·00	21·0	4·90
VII (¼ A) (−ve)	4	8·4	1·97	0·18	0·05	2·64	21·7	2·74
VIII (1 L) (−ve)	4	7·8	2·07	0·12	0·01	2·79	7·7	1·76
Wistar rats on normal diet:								
IX	4	4·2	0·68	0·26		—	—	6·20
X	4	4·3	—	0·20		—	—	4·70

gave only 4·4 mg. of a bromide insoluble in ether and benzene which decomposed above 240° without melting, and which may therefore be regarded as consisting chiefly of octa- or deca-bromide though the amount was too small for a bromine determination. The amount was, however, less than one-tenth of that isolated from the rats which had received the supplement of linoleate or arachidonate. The muscle phospholipin of the negative control rats also gave a small quantity

of insoluble bromide (6·2 mg.). No benzene-insoluble bromide was precipitated on brominating the fatty acids of the neutral fat.

The hexabromide (M.P. 202°) isolated from the livers of rats fed for 257 days on a fat-free diet and described in our previous communication [1938] was not identified, but the period of feeding with the fat-free diet in the present work was only 215 days and the longer period was probably necessary to ensure the complete exhaustion of the arachidonic acid.

After 215 days of the fat-free diet, small amounts of arachidonic acid still remained in the phospholipin of the liver and muscle tissues. In our previous work [1938] we found that after 257 days the liver was completely denuded of this acid.

In the present experiment the total increase in weight of 8 rats on the fat-free diet from the 163rd day to the 215th day on which they were killed, was only 28 g.: an average of less than 0·5 g. increase per rat per week. Since at the end of 31 weeks of the fat-free diet, the proportions of neutral fat and phospholipin were normal in liver, kidney and muscle tissue, there is no evidence that there is any failure in the processes by which these substances are formed in the fat-starved rat although the power to increase in weight has been lost. It must be remembered that during this period of depletion the rats have a constantly diminishing content of arachidonic acid, and until this is completely exhausted it cannot be assumed that this acid plays no part in the metabolic processes which are taking place in the organism. Our results support the view of Sinclair [1938] who studied the phospholipin metabolism of rats fed with elaidic acid and concluded that phospholipins containing only oleic and the saturated fatty acids serve in transporting fat and favour the combustion of fatty acids in the body.

In the rats fed with supplements of linoleic and arachidonic acids evidence of the occurrence of arachidonic acid was obtained in the neutral fat of the liver but considerably more occurred in the phospholipin fraction. In muscle fat

Table 2. *The weights of benzene-insoluble bromide derived from the arachidonic acid in phospholipin and neutral fat*

The amount of arachidonic acid is probably represented approximately by three times the weight of the benzene-insoluble bromide, precipitated in benzene solution and washed.

		In phospholipin			In neutral fat	
		Wt. of bromide			Wt. of bromide after washing with benzene mg.	% Br
Group	No. of rats	(*a*) As ppted in benzene solution mg.	(*b*) After washing with benzene mg.	% Br in (*b*)		
			Liver			
I (−ve)	8	—	4·4	—	0	—
II (Linoleate 60 days)	7	62·7	43·5	66·1	12·5	—
III (Arachidonate 39 days)	6	105·6	68·8	67·4	<1	—
IV (Arachidonate 47 days)	8	61·3	44·7	64·6	17·0	69·5
			Muscle			
I		—	6·2	—	<1	—
II		—	4·2	—	20	68·4
III		28·0	25·0	68·2	5	—
IV		—	8·0	—	10	—

arachidonic acid occurred both in neutral fat and phospholipin. The amounts of arachidonic acid are much greater in the phospholipin from liver than in that from muscle.

In our second experiment, we investigated three groups, each containing 4 rats; after 163 days on the fat-free diet, the rats in each group received per rat a supplement of methyl arachidonate consisting of 1 drop daily, 1 drop every other day and 1 drop on every 4th day in the three groups respectively for a period of 5 weeks, thus constituting doses of 1, 0·5 and 0·25 drop respectively for the rats in each group. The rats then again received only the fat-free diet until they were killed. A fourth group of 4 rats received a daily supplement of 1 drop linoleate during the 5-week period.

In order to determine the degree of fatness, and to avoid subjective impressions, we extracted and weighed the fat from the various tissues making in all about 80% of the total weight. Neglecting the paws, head and tail we divided each group of rats into 6 fractions: (1) livers, (2) muscles from the hind limbs, (3) kidneys, (4) skin, (5) carcases, and (6) omentum with viscera. The carcase fraction included bones, muscles, thorax and the abdominal deposits of fat lying round the kidneys and on the back muscles.

On opening the abdomens of the rats which had received the sub-curative doses of arachidonate (0·5 and 0·25 drops) the animals appeared particularly well fattened, indeed they seemed to be loaded with fat and this appearance was confirmed by the weights of fat extracted. The figures are set out in Table 3, groups VI and VII.

The effects of small doses of the unsaturated acids on fat storage

In the liver. The addition of small doses of the essential unsaturated acids to the diet did not appear to have any very marked influence on the storage of liver fat. Whereas the percentages of fat obtained from the two similar groups of rats fed on a normal diet were 3·6 and 5·0, the percentage in the ten groups examined in our two experiments lay between 3·2 and 5·0. The only variation which might be regarded as significant was in the proportion of phospholipin to neutral fat in the three groups which had received respectively doses of 1, 0·5 and 0·25 drop arachidonate and had then returned to the fat-free diet. In these the percentage of phospholipin was between 30 and 40, being lowest in the livers of the "0·5 drop" rats.

In all the other groups examined the phospholipin percentages lay approximately between 45 and 55%. The return to a fat-free diet after receiving for 5 weeks daily doses of arachidonate seemed to be associated with a diminution in the liver phospholipin content but not in that of the total lipin.

In muscle tissue. In three of the four groups now under investigation the proportion of total lipoid matter in the muscles was between 4·5 and 4·9%, but in the rats which had received the 0·5 drop doses, the muscles contained 7·4% of total lipin and showed a striking increase in both phospholipin and neutral fat contents. The proportion of muscle fat in all four groups seemed distinctly greater than in those which had continued to receive the doses of unsaturated acids until they were killed. In all groups the percentage of phospholipin varied from 9·0 to 12·9, agreeing with the values found in our first experiment.

In the skin. The proportion of total lipin is greatest in the rats which had received the 0·5 and 0·25 drop doses. Calculated on the fat-free dry weight the skin of the "0·5 drop" rats gave 62·2%, the "0·25 drop" 51·9% and the "1 drop" rats 43·0% total lipin. It is again seen that the skins of the rats receiving the smaller doses are exceptionally well fattened as compared with

those of the rats which had received the 1 drop doses of arachidonate, and that as in the case of the muscles the proportion of skin fat is greatest in the rats which have received the 0·5 drop doses. This high percentage of fat in rats which after a short period of dosing had for nearly 6 weeks been living on the fat-free diet is striking.

In the kidney. The amount of fat extracted from the kidneys of each group of 4 rats was very small and was not separated into neutral fat and phospholipin nor was the percentage of phosphorus in the total lipin determined. The percentages of total lipin in the rats which had received the doses of 1 and 0·5 drop arachidonate followed by the fat-free diet were respectively 5·0 and 4·9 (cf. Table 1), agreeing with the values found in the groups of two normally fed Wistar rats (4·7 and 6·2 %); on the other hand the values in the groups of rats which had received the doses of 0·25 drop arachidonate and 1 drop linoleate were 2·7 and 1·7 %, i.e. less than in any other group investigated. No obvious correlation between the amounts of fat and the experimental conditions was visible and we felt that in order to investigate the kidney lipin satisfactorily, it would be necessary to use a larger number of animals in each group, so that more material would be available.

In the carcase. Quite abnormally large amounts of lipin were extracted from those rats which after receiving the 0·5 and 0·25 drop doses of arachidonate had been again transferred to the fat-free diet. Unfortunately in the working up of the fat from the corresponding group of rats fed with the 1 drop doses a small amount of fat was lost, not more than 5 % of the whole. Compared with two Wistar rats which had throughout received a complete diet, the rats which had received small doses of arachidonate for 5 weeks before being returned to the fat-free diet showed abnormal fattening in the inverse order of the amounts of arachidonic acid that they had received. Thus the carcases of the two groups of Wistar rats fed on a complete normal diet contained 35·7 and 38·0 parts total lipin for each 100 g. of fat-free dry tissue—compared with 40·8, 54·1 and 55·2 % for the rats which had respectively received the 1, 0·5 and 0·25 drop doses.

In the viscera. Since no fat had been contained in the diet of any of these rats, the omentum with the spleen, stomach, intestines and their contents were removed, weighed and the total lipin extracted and separated into neutral fat and phospholipin.

The superfatted condition of the rats which had only received for 5 weeks the subcurative doses of arachidonic acid and had then subsisted for nearly 6 weeks again on the fat-free diet, was a great surprise to us.

In Table 3 are set forth the total amounts of neutral fat and phospholipin extracted from the various groups. The wet weight of the tissues had not been recorded in all cases, and only in the group receiving the 0·25 drop doses, are the wet weights of all the tissues examined, available. It will be seen in this case that the latter represented 82 % of the total weights of the rats at the time of killing.

The crude phospholipin separated from carcase, skin and viscera was always small in amount and very impure since the phosphorus percentages lay between 1 and 2. The amounts of phospholipin from these organs shown in Table 3 have been recalculated and are given as the amounts which would contain 3·8 % P. The smaller adjustment necessary has also been made in the figures for liver, kidney and muscle.

During the 5 weeks period of dosing, the 4 rats fed with the 0·25 drop doses had made a total gain of only 9 g. in weight and another 8 g. during the following 38 days on the fat-free diet. But as shown in Table 3 their tissues contained

Table 3. *Showing total amounts of neutral fat and phospholipin in the 4 groups*

A = arachidonate L = linoleate

	Group V Dose for 5 weeks 1 drop A daily 14 days – ve			Group VI Dose for 5 weeks 0·5 drop A daily 38 days – ve		
	Wet wt. tissue	Neutral fat	Phospho-lipin	Wet wt. tissue	Neutral fat	Phospho-lipin
	g.	g.	g.	g.	g.	g.
Liver	35·0	0·7	0·38	35·5	0·7	0·27
Sample of muscle	52·5	2·4	0·18	52·5	3·5	0·34
Carcase	—	32·9 +	0·61	—	45·6	2·00
Kidney	7·8	0·6	0·02	8·7	0·2	0·03
Skin	—	18·8	0·27	109·6	22·4	0·27
Viscera	79·4	8·1	0·11	83·9	8·9	0·22
		63·5 +	1·57		81·3	3·13
Total wt. fat + phospholipin		65·07			84·43	

	Group VII Dose for 5 weeks 0·25 drop A daily 40 days – ve			Group VIII Dose for 5 weeks 1 drop L daily 12 days – ve		
	Wet wt. tissue	Neutral fat	Phospho-lipin	Wet wt. tissue	Neutral fat	Phospho-lipin
	g.	g.	g.	g.	g.	g.
Liver	32·9	0·9	0·44	35·2	0·9	0·50
Sample of muscle	58·6	2·6	0·23	57·0	2·4	0·30
Carcase	361·0	48·7	1·06	—	32·4	0·56
Kidney	8·4	0·4	0·04	7·8	0·1	0·01
Skin	109·8	19·0	0·14	—	19·0	0·08
Viscera	84·0	7·6	0·14	—	7·2	0·11
	654·7	79·2	2·05		62·0	1·56
Total wt. fat + phospholipin		81·25			63·56	

Table 4. *Showing total weight of 4 rats in each group*

Dosing started on 163rd day after weaning and ended on 198th day and rats then returned to fat-free diet.

Rats in Group V killed on 212th day—in Group VI on 236th day, in Group VII on 238th day and in Group VIII on 214th day.

	Group V "1 A"	Group VI "½ A"	Group VII "¼ A"	Group VIII "1 L"
	g.	g.	g.	g.
On 163rd day	677	726	778	660
On 198th day	789	783	787	709
When killed	812	795	795	723
Gain in wt.:				
During dosing period	112	57	9	49
During subsequent period on fat-free diet (14 days)	23	(38 days) 12	(40 days) 8	(12 days) 14
Total gain	135	69	17	63

about 16 g. more fat than those of the rats which had received the 1 drop doses of arachidonic or linoleic acid for a similar period. Their total increase in weight therefore corresponds almost exactly to the excess of fat put on during the 5 weeks period of dosing and the subsequent 40 days on the fat-free diet. The effect of the smallest doses of arachidonate is therefore to store up fat in the fat deposits and there is no evidence that there has been any formation of new

tissue. It may be noted that in this case almost the whole of the increased weight of fat is stored in the fat deposits of the carcase.

The "0·5 drop" rats contained 3 g. less fat in their carcases than the "0·25 drop" rats, but muscle, skin and viscera contained more fat, so that there was in all about 18 g. more fat in the tissues examined than in those of the rats which had received the 1 drop doses of arachidonate or linoleate. The total weight increase in this group is however 69 g. of which 57 g. were put on during the period of dosing and 12 g. in the 38 days on the fat-free diet which supervened. About 50 g. of tissue which is not fat have been added with the increase in the dose of arachidonate and must presumably have represented an increase of new tissue provided by the formation of new cells.

The 4 rats receiving the 1 drop doses of arachidonic acid showed a weight increase of 112 g. during the dosing period, and of 23 g. in the following 14 days on the fat-free diet. These rats were well fattened but did not show the excessive fattening characteristic of the rats receiving the smaller doses.

These results indicate that the first effect of the arachidonate is to load up the connective tissue cells with lipoid matter especially in the perinephric fat of the carcase and that subsequently this excess of fat disappears as active growth occurs in the tissues.

The total weights of the rats in the three groups receiving the doses of arachidonate did not vary very widely (Table 4) and the total amounts of neutral fat and phospholipin extracted from the different groups may therefore be regarded as roughly comparable. The total phospholipin is markedly higher in the rats which have received the 0·25 and 0·5 drop arachidonate doses, and this is almost entirely made up of the excess in the carcase. While the excessive filling up of the fat depots is taking place the phospholipin increases proportionately more than the neutral fat, providing fresh evidence that the phospholipin is concerned in the transport of fat to the fat depots.

It is also interesting that in the lipoid matter from the livers of the rats fed entirely on the fat-free diet and in those which have received doses of the unsaturated acids continuously until they were killed the phospholipin forms from 42 to 52% of the total lipoid matter. However, in the rats which after a long period of fat starvation received for 5 weeks doses of arachidonate and then returned to the fat-free diet, the phospholipin content of the total lipoid matter varied from 30 to 40%.

A comparison of the two groups of rats which received 1 drop doses for 39 days and were then killed with those receiving doses for 35 days and then returning for 12 days to the fat-free diet is particularly interesting. Unfortunately only the figures for liver and muscle are available. These show a marked fall in liver phospholipin for the rats transferred to the fat-free diet and a marked increase in the percentage of neutral fat in the muscle tissue.

The proportion of arachidonic acid in the fat deposited

The proportion of arachidonic acid in the tissues of the rat as indicated by the weights of benzene-insoluble bromide formed on bromination varied greatly according to the amounts contained in their food. In rats which had been fed continuously on a normal diet containing adequate amounts of fat with the essential acids, the proportion of arachidonic acid in the lipoid matter extracted from the various tissues was high, especially in the liver. The amounts of fat derived from the small weights of kidneys available were so small that generally we did not attempt to analyse them further.

Table 5 shows the effects of the various diets on the amounts of benzene-insoluble bromides obtained per g. of total lipin. The figures given are certainly comparable and when multiplied by 2·9 probably give a fair indication of the weights of arachidonic acid in the lipin.

Table 5. *The influence of diet on the proportion of arachidonic acid found in the total lipoid substance (fat and phospholipin) from rat tissues*

The figures given represent the number of mg. of octabromide, precipitated on bromination of the fatty acids in benzene solution, the precipitate being subsequently extracted with benzene. The actual amounts of arachidonic acid are probably nearly equal to the figures given multiplied by 3.

	1		2		3
	Rats fed 163 days on fat-free diet, then received supplements of unsat. acids until they were killed		Rats fed 163 days on fat-free diet, then received supplements of unsat. acids for 35 days followed by fat-free diet		Rats fed for 215 days on fat-free diet and then killed
Liver	1 A (39 days)	44·2	1 A (14 days)	12·6	2·2
	1 A (47 „)	33·4	½ A (31 „)	4·4	
	1 L (60 „)	24·6	¼ A (40 „)	5·3	
			1 L (12 „)	8·5	
Muscle	1 A (39 days)	8·6	1 A (14 days)	3·9	1·7
	1 A (47 „)	3·9	½ A (31 „)	3·4	
	1 L (60 „)	5·8	¼ A (40 „)	3·9	
			1 L (12 „)	2·3	
Carcase			1 A (14 days)	2·8	
			½ A (31 „)	1·2	
			¼ A (40 „)	1·5	
			1 L (12 „)	0·9	
Skin			1 A (14 days)	1·0	
			½ A (31 „)	0·13	
			¼ A (40 „)	0·05	
			1 L (12 „)	0·05	

1 A = 1 drop arachidonic acid daily.
½ A = 1 drop arachidonate every other day.
¼ A = 1 drop arachidonate every fourth day.
1 L = 1 drop linoleate daily.
The number of days in brackets in col. 2 indicates the number of days the rats were returned to the fat-free diet before being killed after the period of dosing was ended.

The livers of two groups of rats kept for 163 days on a fat-free diet and then given a daily dose of 1 drop arachidonate for periods of 39 and 47 days respectively, gave 44·2 and 33·4 mg. octabromide per g. of total lipin. These contrasted with a group which after the 163 days on the fat-free diet had received for 35 days a daily dose of 1 drop arachidonate and were then again given the fat-free diet until they were killed 14 days later: here the octabromide precipitate had fallen to 12·6 mg. A similar result was obtained with linoleic acid; after 163 days of fat-free diet, 8 rats received daily 1 drop linoleic acid for 60 days before being killed: their liver fat gave 24·6 mg. octabromide per g. fat. The liver lipin of rats fed with similar doses for 35 days and then kept for 12 days on the completely fat-free diet yielded only 8·5 mg. octabromide per g. lipin. The livers of the rats which had received the 0·5 and 0·25 drop doses of arachidonate followed by the return to the fat-free diet for 31 and 40 days respectively gave 4·4 and 5·3 mg. arachidonic acid per g. total lipin, but in those kept for 215 days on the fat-free diet the comparable amount of octabromide had fallen to 2·2 mg. We have previously shown that with long-continued fat-free feeding, no arachidonic acid was detected.

In muscle lipin somewhat the same relationship holds but the differences between the amounts of octabromide in the different groups are much less and the return to the fat-free diet after receiving the unsaturated acids seemed to exert much less influence than in the liver.

For the carcase and skin lipins unfortunately the figures for only one group are available, the data for these not having been determined in the first experiment.

The percentages of bromine in the benzene-insoluble bromide from the carcase fat of the rats which had received the 0·25 and 0·5 drop doses were abnormally low. Thus the figures for the benzene-insoluble bromide from the carcase phospholipin of the rats dosed with the 1, 0·5 and 0·25 drops arachidonate were respectively 1 A, 6·5 mg. (not analysed): ½ A, 44·5 mg. (63·6 % Br): ¼ A, 34·5 mg. (61·7 % Br) whilst for the neutral fat the figures were 1 A, 94·2 mg. (67·4 % Br): ½ A, 11·2 mg. (not analysed): ¼ A, 39·9 mg. (70·2 % Br). Theory for arachidonic octabromide requires 66·7 %. There was no obvious reason why the two phospholipin results gave such low figures. Possibly some of the dihydro-arachidonic hexabromide previously described by us [1938] was present.

In the skin fat from the same group of rats, only traces of benzene-insoluble bromide were precipitated although the dry scurfy condition of the ankles of the rats which had received the one drop doses of arachidonate had been completely cured.

A comparison of the four closely similar groups of 4 rats which received the doses of unsaturated acids for 35 days, indicated that the rats fed with daily doses of one drop linoleate contained in the total tissues examined (approximately 70 % of the weights of the rats) less than half as much arachidonic acid as did those fed with the daily dose of one drop arachidonate. It is unfortunate that similar figures were not available for the rats fed only on the fat-free diet but in the earlier experiments only the liver and muscle fat was analysed. A group of 3 rats which had been kept for many months on a fat-free diet and which should have provided the necessary comparison was not available owing to accidental circumstances.

The total amounts of benzene-insoluble bromide obtained from the groups of rats fed with the 0·25, 0·5 and 1 drop doses of arachidonate respectively were not widely different. They were only slightly higher in the rats which had received the whole drop doses. This fact might in part have been contributed to by the fact that the "1 drop" rats were killed rather less than 2 weeks after receiving their last dose of the acid whereas 5 to 6 weeks had elapsed after the dosing period before the "0·5" and "0·25 drop" rats were killed and during the longer period on the fat-free diet, more of the arachidonic acid would presumably have disappeared.

The interpretation of the results based on the estimation of arachidonic acid as the benzene-insoluble bromide is complicated by the fact that in our experience the solubilities of the octabromides of the naturally occurring arachidonic acid and of that obtained by debromination of the arachidonic-octabromide differ considerably when the precipitation is carried out in very dilute solution. Precipitation of the insoluble bromide of the natural acid is not very much affected even when conducted in very dilute benzene solutions but the bromide of the debrominated acid is considerably more soluble. On the other hand when precipitating in ethereal solution, the bromide of the recovered acid is more insoluble than that of the natural acid. As we did not discover this until late in the investigation, it was not possible to work out a more satisfactory method and we realize that whereas in the cases of the carcase and skin the

Table 6. *Showing the mg. of benzene-insoluble bromide isolated from the tissues of rats*

After 163 days on fat-free diet the rats had received doses of 1, 0·5 and 0·25 drop arachidonate and 1 drop linoleate respectively and had then been transferred to the fat-free diet for varying periods before being killed. The tissues extracted formed about 70% of the total weights of the rats.

Supplement fed to rats	1 drop linoleate		1 drop arachidonate		0·5 drop arachidonate		0·25 drop arachidonate	
	Neutral	Phospholipin	Neutral	Phospholipin	Neutral	Phospholipin	Neutral	Phospholipin
Liver	3	9·2	6	6	2	3·3	2	7
Sample of muscle	1	4·8	7	2	5	8	7	4
Carcase	31	5·5	94	7	11·2	44·5	39·9	34·5
Skin	0	1	16	2·8	0	3	0	1
Total in:								
Neutral fat	35		123		18·2		48·9	
Phospholipin	20·5		17·8		58·8		46·5	
Total	55·5		140·8		77·0		94·4	
Intake of arachidonic acid during feeding			1762		958		480	

The weights of benzene-insoluble bromide × 3·0 give an approximate indication of the amounts of arachidonic acid present.

percentage of arachidonic acid in the fat is low, considerable quantities of the debrominated acid might be stored and escape detection.

It is possible that on debromination the arachidonic acid is partially or wholly isomerized. As shown in the preceding communication the debrominated arachidonic acid can certainly be utilized in the body; feeding experiments in which the natural and recovered acids may be compared are already in progress.

It appears likely that the inclusion of arachidonic acid in a phospholipin is a necessary stage of the process of increasing the fat in the storage cells. In the most highly fattened rats (the 0·5 and 0·25 drop doses) a large proportion of the carcase arachidonic acid was contained in the phospholipin fraction. This may be due to some part played by the arachidonic acid in the transport of fat to the depot cells or possibly in the passage of the fatty acids through the cell membranes into bodies of the connective tissue cells. It is possible that in this process the incorporation of arachidonic acid into the kephalin molecule may play a part.

Since nearly as much arachidonic acid was detected in the tissues of the "0·25 drop" rats as in those of the "0·5 drop" rats, the increase in tissue weight other than that of fat, which was shown only by the "0·5 drop" rats probably did not depend wholly on the level of arachidonic acid in the tissues. In the "1 drop" group the amount of arachidonic acid contained in the phospholipin fraction was markedly less than in the "0·25" and "0·5 drop" groups.

In order that growth should take place as shown by an increase of weight of the animal much greater than that represented by any increase in the weight of fat, apparently in addition to the storage of arachidonic acid in the body phospholipin, a large excess of arachidonic acid is necessary and since it was not detected by estimation as the benzene-insoluble bromide, it is unlikely that it was present as the naturally occurring acid. In the "1 drop" rats, which gave no evidence of excessive fattening but showed marked increase in tissue weight we found no evidence of storage of a large proportion of the 1762 mg. arachidonic acid which they had received, and it was therefore probably not present in the

body as the natural arachidonic acid. Possibly arachidonic acid is oxidized in a chain of reactions associated with some vital process or has been converted into some other substance essential for cell proliferation.

It is perhaps of interest in this connexion to recall that Evans *et al.* [1934] found that in the absence of the highly unsaturated acids, even when vitamin E was supplied male rats were sterile and ovulation in the female was hindered.

Two distinct processes in which arachidonic acid plays an essential part appear to us to be indicated by our results:

(1) This acid plays some essential part in the process by which the connective tissue cells of the fat depots are first loaded up with fat. The effect is most apparent in the abdominal depots. It is possible that the inclusion of the arachidonic acid in the kephalin molecule might play a part in this process, although there is no evidence on this point.

(2) After the connective tissue cells have been filled up with fat, growth takes place. This is associated with a disappearance of the excessive fat deposits and the intake of a large excess of arachidonic acid which is converted into other products and is not stored as such in the body.

Cholesterol metabolism

In view of the fact that the highly unsaturated acids occur in the blood largely in combination with cholesterol, we determined the unsaponifiable matter in the neutral fat extracted from the organs of the four groups of rats which had received the doses of unsaturated acids for 35 days before being returned to the fat-free diet. These results are shown in Table 7.

Table 7. *Showing weights of unsaponifiable matter in the total lipin of rats fed on fat-free diet and receiving doses of unsaturated acids*

	Group VIII	Group V	Group VI	Group VII
Dose	1 drop linoleate mg.	1 drop arachidonate mg.	0·5 drop arachidonate mg.	0·25 drop arachidonate mg.
Liver	169	145	257	192
Muscle	67	59	75	111
Skin	524	574	634	765
Carcase	472	484	504	491
Kidney	14	39	38	47
Total	1246	1301	1508	1606
Total weights of phospholipin in same organs	1·45	1·46	2·91	1·91

The amounts of cholesterol are somewhat greater in the rats which have received the smaller doses of unsaturated acids.

The urine of the rats receiving the 0·25 and 0·5 drop doses was tested for acetone with negative results.

Note on the estimation of arachidonic acid

The methyl arachidonate used in the feeding experiments described in the previous communication was obtained by debromination of the octabromides prepared by brominating the crude fatty acids of suprarenal fat in solution in ether or benzene. So far as was known the arachidonic acid prepared in this way was identical with the naturally occurring acid. Since arachidonic acid contains 4 double bonds, it is capable of occurring in 16 geometrically isomeric forms. The possibilities of rearrangement both on bromination and debromination are therefore considerable.

Brown & Ault [1930] found differences in the melting points of the methyl esters of the octabromides derived respectively from ox and hog brains which they suggested might be due to the formation of isomeric bromides. Bosworth & Sissons [1934] showed that at least three isomeric octabromides were formed when the unsaturated acids separated from butter fat were brominated in ethereal solution and washed with ether. They isolated three bromides: (1) insoluble in petrol and soluble in ether M.P. 162°; (2) insoluble in ether but soluble in benzene M.P. 208°; (3) insoluble in all three solvents M.P. 257°. The same three bromides were also obtained by brominating in cold petrol solution, but with melting points 5 to 10° lower than those obtained by bromination in ethereal solution. All three bromides contained the theoretical amount of Br, on debromination gave an acid of mol. wt. 306·5 and I.V. 332·3 and on reduction arachidic acid, M.P. 74.

Experiments were made to determine the relative amounts of bromide precipitated when the debrominated arachidonic acid was brominated in 2% solution in various solvents (Table 8). The results show that when brominated in petrol solution as much as 85% of the theoretical amount of octabromide is precipitated of which more than 80% is ether-soluble. In preparing the octabromide obtained from a mixture of unsaturated acids, it should be advantageous to brominate in petrol solution as so high a percentage of octabromide is precipitated. In the absence of linoleic acid, which also gives a petrol-insoluble bromide, this should certainly prove the best method of preparation. Bromination in benzene gives little more than half the yield obtained by brominating in ethereal solution and is not therefore an economical method of recovering the acid. It does however yield an octabromide free from possible hexabromides.

Table 8. *Showing effect of solvent on amount of octabromide precipitated from 2% solutions of arachidonic acid obtained by debromination*

Weight of arachidonic acid g.	Solvent	Volume ml.	Weight insoluble bromide g.	% of theoretical amount of octabromide
0·3044	Light petroleum B.P. 50–60	15	0·7796	85·1
0·2866	Ether	15	0·2566	29·1
0·4408	Ether	20	0·3896	29·3
0·2958	CCl_4	15	0·2618	29·4
0·3186	Benzene	15	0·3186	16·3

The bromides precipitated in petrol and in ethereal solutions, were each heated with 70 ml. ether for 1 hr., the solutions being then cooled to laboratory temperature and decanted. This process was repeated nine times, the amounts of the precipitates dissolved being given below.

	Amounts dissolved from ppt. formed in petrol solution mg.	Amounts dissolved from ppt. formed in ethereal solution mg.
First extraction	530·6	60·3
Second extraction	61·4	48·0
Third to sixth	55·6	55·6
Seventh to tenth	23·2	24·8
Total amount extracted	670·8	188·7
Residue	91·6	204·6

The residues were then each extracted three times with 50 ml. benzene, when 71·6 mg. of residue from the precipitate from the petroleum solution and 60·4 mg. residue from the precipitate from the ethereal solution remained.

302 mg. bromide from the benzene solution were then extracted eight times with benzene, being heated each time with 15 ml. benzene for 1 hr.

The first three extractions removed 108 mg. substance soluble in benzene: the next five extracts contained 25 mg. leaving 168·8 mg. residue.

From these figures the proportions and ether- and benzene-soluble bromides may be calculated as percentages of the theoretical amounts of octabromide.

In ...	(1) Petrol %	(2) Ether %	(3) CCl_4 %	(4) Benzene %
Total ppt.	85·1	29·3	29·4	16·3
Ether-soluble fraction	70·2	14·3	—	7·3
Benzene-soluble fraction	6·2	5·3	—	9·1
Benzene-insoluble fraction	8·7	9·7	—	—

From these figures it seems probable that the precipitated bromides differ chiefly in the proportion of ether-soluble bromide which they contain. At least four isomeric bromides seem to be present:

(1) Petrol-insoluble and ether-soluble.
(2) Petrol- and ether-insoluble.
(3) Ether-insoluble: sparingly soluble in benzene.
(4) Very slightly soluble in benzene.

Ault & Brown [1934] found that when methyl arachidonate was brominated in ether and the precipitate washed with ether, the weight of bromide precipitate, though only about 26% of the theoretical amount, bore a constant ratio to the original weight of acid brominated. Further, when the C_{20} fractions of the methyl esters of the original unsaturated acids were brominated and the arachidonate content calculated from the I.V. the proportion of bromide insoluble in ether was similar to that obtained from the debrominated acid. They considered therefore that the most probable estimate of the amount of arachidonate present was obtained by multiplying the weight of bromide precipitated in ether by the factor 1·29.

In estimating the proportion of arachidonic acid present in the small amounts of fat derived from the organs of the rat, the fractionation of the methyl esters appeared to us impracticable as a stage in the process. It was obviously more convenient to brominate the mixed unsaturated acids or if the process were not affected by the presence of the saturated acids to brominate the mixed fatty acids at once. With regard to the solvent used for bromination, ether seemed to us undesirable in the present investigation because, although the ether-soluble bromides of oleic and linoleic acids would be removed, the benzene-soluble hexabromides would be weighed with the octabromide of arachidonic acid. It was unlikely that linolenic hexabromide would be present but the presence of the hexabromide of the C_{20} acid previously described by us [1938] was possible and in another series of experiments we had isolated a hexabromide of a dihydroxy-C_{20} acid also soluble in benzene. In order to eliminate as far as possible these benzene-soluble hexabromides, we decided to carry out the brominations in benzene solution.

As the solid bromide precipitated in ether or in benzene represented only a fraction of the theoretical amount of octabromide, experiments were made to

establish the constancy of the fractions precipitated and to determine a factor which, when multiplied by the weight of insoluble bromide precipitated, would give the weight of arachidonic acid present.

Two procedures were adopted: (1) the precipitates were left 48 hr. in the cold room, filtered on weighed filter papers, washed with 5–7 ml. ether and dried in a desiccator to constant weight; (2) the bromine was added to the solution of the acid in a weighed centrifuge tube which was left for 48 hr. in the cold room, then centrifuged and the residue again centrifuged three times with 5 ml. ether and dried to constant weight. In some of the experiments a considerable excess of palmitic and oleic acids was added to the solution to give conditions comparable with those occurring when the fatty acids of the rats' tissues are brominated.

Three samples of acid were used in these estimations:

(*a*) the acid obtained by debrominating octabromide prepared from suprarenal fat and containing 68·3 % Br;

(*b*) a mixture of oleic and arachidonic acids prepared from a sample of methyl esters of unsaturated acids which had been purified by molecular distillation and which from the I.V. contained 41·34 % arachidonic and 58·66 % oleic acids;

(*c*) the crude fatty acids obtained from suprarenal fat.

The two chief difficulties encountered were: (1) the amounts of precipitates formed from similar quantities of the debrominated acid and of that present in the natural fat showed marked differences especially in dilute benzene solution; (2) the solubility coefficients of the "insoluble" bromides were sufficient to cause serious discrepancies in very dilute solutions.

In order to purify the mixture of fatty acids without using either high temperature distillation of the methyl esters or a process involving the addition and removal of bromine, we enlisted the help of Mr Carr (British Drug Houses) and Dr Mead undertook to distil the methyl esters of the unsaturated acids separated from the acids of suprarenal fat by the Pb salt-alchol process I.V. 169·5; the fractions obtained were as follows:

	Temperature ° C.	I.V.	Weight g.
1	65	—	0·61
2	75	—	6·55
3	85–90	—	9·61
4	85–90	183·2	12·38
5	85–90	178·6	12·52
6	90	214·0	5·9
7	100–110	222·3	2·08
8	120	—	1·50
9	120–160	99·6	4·80

Fractions 3, 4 and 5 distilling at 85–90° were colourless liquids remaining completely liquid after long standing in the cold room. In fraction 6, some solid separated and fraction 7 solidified completely. Fraction 4 was saponified: from its I.V. it consisted of 41·34 % arachidonic mixed with oleic acid, and this mixture of acids was used for the bromination experiments.

A comparison of the factors arrived at when using the three different samples of acid is given below. It will be seen that with the debrominated acid the amount of bromide precipitated in 0·3 % solution is much less than with the acid purified by molecular distillation and that in the former case the factors are very large and irregular.

Factor, calculated from weight of insoluble bromide precipitated in ethereal solution from acids purified by molecular distillation, and washed three times with benzene.

Arachidonic acid %	A Debrominated acid	B Acids from methyl esters after molec. dist.
1·2	$F=2·15$	$F=1·90$
0·3	1·90	2·14
0·1	3·08	2·29

The solubilities of the bromides obtained by brominating the suprarenal fatty acids and the debrominated arachidonic acid in benzene solution were compared. Four extractions, each with 15 ml. benzene, dissolved 133 mg. from 302 mg. of the latter, i.e. 44 %, whereas in the case of the bromide from the

Table 9. *Estimation of Factor F where* $F=\frac{\text{Wt. arachidonic acid}}{\text{Wt. insoluble bromide}}$.

Column A contains results where weighed amounts of debrominated arachidonic acid were used: in B, a mixture of oleic and arachidonic acids purified by molecular distillation of the methyl esters was used and the amount of arachidonic acid calculated from the I.V. of the mixture: in C the crude fatty acids from suprarenal fat were used and the amount of arachidonic acid calculated as 1·2 times the weights of bromide precipitated in 1–2 % ethereal solutions.

% arachidonic acid	Insol. bromide pptd in ether			Insol. bromide pptd in benzene and washed with ether		
	A	B	C	A	B	C
1·9–2·0 %	$F=1·13$	—	—	$F=2·14$	—	—
	1·39			2·47		
	1·24			2·27		
	1·12			2·39		
				2·23		
				2·66		
				2·32		
				2·24		
				2·03		
1·0–1·2 %	1·32†	1·26	1·30	2·83	2·88	2·0
0·3 %	1·44†	1·44	—	8·35	3·40	—
	1·39			7·12		
				10·10		
				13·00		
				7·42		
				9·75		
				6·73*		
				5·52*		
				5·39*		2·1
0·2 %	—	—	—	—	—	1·8
0·1 %	1·76†	2·16	—	1·14?	2·00	—

* Oleic and palmitic acids were added to the solution.

† A mixture of 59 % oleic and 41 % arachidonic acid used to correspond with acids obtained by molecular distillation.

suprarenal acids, 72 mg. out of 282 mg. were dissolved, i.e. 26 %. The precipitates were heated in each extraction for 1 hr. with 15 ml. benzene, the solutions cooled to laboratory temperature and decanted. The bromide from the debrominated acid was therefore more soluble in benzene than that obtained from the natural acid. The percentages of bromine determined by Dr Weiler were:

Suprarenal acid, benzene-insol. fraction 67·3 %: benzene-sol. 64·9 %.
Debrominated acid, benzene-insol. fraction 66·4 %: benzene-sol. 67·0 %.
Theory for arachidonic octabromide = 67·7 %.

The bromide formed in benzene solution contains therefore at least two bromides, one removed readily by extraction with hot benzene and the other only slightly soluble in benzene. Whilst bromination in benzene solution gives a fair approximation of the amount of the naturally occurring arachidonic acid, it is not satisfactory in determining the amount of the acid obtained by debromination of the octabromide; the two acids appear to differ both in the relative solubilities of their ether-soluble and benzene-soluble bromides.

In the case of the natural acid, the ratio of the ether-insoluble to the benzene-insoluble bromide differs considerably from that obtained with the debrominated acid. And whereas, in the case of the natural acid, the amount of bromide precipitated in benzene solution is only slightly less on greatly diluting the solution, the amount of that precipitated in ether diminishes markedly on dilution. With the debrominated acid, the reverse is the case, here the amount of ether-insoluble bromide is only slightly affected on dilution whilst that of the benzene-insoluble fraction rapidly diminishes.

Thus the ratio of the weights of insoluble bromides precipitated in ether and benzene from the natural acid were in 1% solution 2·12 and in 0·3% solution 2·35; the corresponding figures for the debrominated acid were 2·2 and 5·2.

From the evidence now obtained it seems probable that during the process of adding and removing bromine, some geometrical isomerization of the acid has occurred. When a mixture of the two forms of the acid is present the estimation presents considerable difficulty: on the whole the discrepancies are probably least when the bromide is precipitated in ether solution and then washed with benzene. It must be emphasized that when thus comparing the amounts of arachidonic acid in small quantities of fat, the brominations should be carried out with approximately the same concentrations of arachidonic acid and the amounts of washing should also be similar. Under these conditions a fair estimate of the relative amounts of this acid may be gained. In our experience the weight of bromide precipitated in ether and three times washed with benzene is approximately equal to half the weight of arachidonic acid present.

Summary

1. The percentages of neutral fat and phospholipin calculated on the wet weight of tissue in the liver, muscle and kidney of rats kept for 215 days on a fat-free diet showed no significant variation when compared with similar figures for rats which after 163 days of fat-free diet had received curative doses of linoleic and arachidonate for 5–7 weeks before being killed.

2. In rats fed for 35 days with doses of arachidonate (1, 0·5 and 0·25 drop) and then returned to the fat-free diet, the liver lipoid substance contained less phospholipin than in those in which the 1 drop doses had been continued.

3. The muscles of rats which had received daily 1 drop doses of arachidonate and linoleate and had then been again fat-starved contained more fat than the muscles of rats which had received similar doses up to the time when they were killed.

4. The tissues, especially skin and carcase, of rats which had received 0·5 and 0·25 drop doses of arachidonate for 5 weeks and had then been again fat-starved were much more fattened than those of rats which had received 1 drop doses of arachidonate or linoleate under similar conditions. The carcases of the 0·5 drop and 0·25 drop rats were also richer in phospholipin.

5. The total weight increase of 17 g. shown by the 4 rats receiving the 0·25 drop doses of arachidonate corresponded almost exactly with the excess

fat found in their tissues. The 4 rats receiving the 0·5 drop doses increased by 69 g. of which only 19 were represented by excess fat. With the "1 drop" rats there was normal fat storage and much increase of weight.

6. Arachidonic acid was absent from the neutral fat of rats fat-starved for 215 days but occurred in very small quantity in the liver and muscle phospholipins. The C_{20} hexabromide (M.P. 202–4°) previously isolated from the liver lipin of rats kept for 257 days on the fat-free diet was not identified.

7. The arachidonic acid stored in the organs of the rats receiving different doses of the unsaturated acids was estimated and compared with the intake of arachidonic acid.

8. The conclusion was drawn that a minimum intake of arachidonic acid is necessary in order that the cells of the fat depots may first be loaded up with fat. Subsequently there is a process of true growth which is accompanied by the disappearance of comparatively large quantities of arachidonic acid.

9. The changes in cholesterol metabolism were also determined.

10. The method of estimating arachidonic acid by precipitation of the insoluble bromide is discussed.

We desire to acknowledge the help given us by Mrs Dolby who carried out estimations of arachidonic acid, by Mr Downer, who was responsible for most of the bromine determinations and by Dr M. G. Macfarlane, in whose laboratory the phosphorus estimations were carried out. We are also much indebted to Dr Mead (British Drug Houses Ltd.) for undertaking the molecular distillation of the fatty acid mixture.

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